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Biochemistry and Physiology of Overwintering in the Mature Larva of the Sunflower Stem Weevil, *Cylindrocopturus adspersus* (Coleoptera: Curculionidae) in the Northern Great Plains

ROBERT R. ROJAS,* LAURENCE D. CHARLET,† ROGER A. LEOPOLD*

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The sunflower stem weevil, *Cylindrocopturus adspersus* (LeConte), overwinters as a mature larva at the base of the stalk and in the root crown of cultivated sunflower plants. Sunflower stalks from fields known to be infested with *C. adspersus* larvae were collected in southeastern North Dakota in October 1991. Larvae from stalks kept outdoors accumulated a high whole-body concentration of trehalose (up to 69 μg/mg wet wt) at the expense of glycogen with the onset of winter followed by a partial reconversion of trehalose to glycogen with the onset of spring. Larvae from stalks acclimated to 0°C also accumulated a high level of trehalose (~69 μg/mg wet wt) with a concomitant decrease in glycogen. Those larvae from stalks kept at 20°C showed an initial sharp increase in whole-body trehalose that then stabilized but at a concentration well below that of larvae acclimated to 0°C. This indicates that there exists in the larva an underlying developmental component to trehalose accumulation which is further enhanced by low temperature (0°C) exposure. The mean temperature of crystallization (T_c) of larvae exposed to outdoor conditions showed an abrupt drop from October (−25.0 ± 1.3°C) to November (−28.2 ± 0.6°C) with a minimum in February (−29.1 ± 0.3°C). The level of trehalose accumulated by the sunflower stem weevil larva is to our knowledge the highest reported in an overwintering insect.

Trehalose Glycogen Temperature of crystallization Insect

INTRODUCTION

Overwintering insects exposed to subfreezing temperatures in their hibernacula have evolved two basic strategies for survival; freeze-avoidance and freeze tolerance (Salt, 1961). Freeze-tolerant insects can survive the formation of extracellular ice within their tissues while freeze-intolerant insects cannot and must avoid freezing to ensure survival.

Many freeze-tolerant and freeze-intolerant insects accumulate low molecular weight polyhydric alcohols (e.g. glycerol, sorbitol) and/or carbohydrates through the breakdown of fat body stores which often is initiated by low temperature exposure (about 5–0°C) (Baust and Miller, 1970, 1972; Rojas *et al.*, 1983). These compounds are thought to provide protection to the organism during exposure to subfreezing temperatures for both the freeze-tolerant and intolerant insects and to freezing for the freeze-tolerant insects. Mechanisms for cryoprotection by these compounds have been proposed and include reduction in osmotic shrinkage and swelling of cells during freezing and thawing, stabilization of proteins and macromolecular structures, reduction in ice content and alteration of ice structure (Baust, 1973; Storey and Storey, 1988; Karow, 1991).

One important aspect of the freeze-intolerant strategy is the colligative depression of the temperature at which the body fluids spontaneously freeze, known as the temperature of crystallization or supercooling point. Survival for this particular group of organisms requires in part, that the temperature of crystallization of their body fluids remains below any low temperatures that they may experience. One mechanism that insects employ to depress their temperature of crystallization is the accumulation of the aforementioned compounds, i.e. sugar alcohols and carbohydrates and in this capacity are referred to as antifreeze agents. These antifreeze agents lower the temperature of crystallization by approximately twice the equilibrium melting point depression (Salt, 1959; MacKenzie, 1977; Gehrken, 1984).

*Biosciences Research Laboratory, USDA-ARS State University Station, Fargo, ND 58105, U.S.A.
†Northern Crop Science Laboratory, USDA-ARS State University Station, Fargo, ND 58105, U.S.A.

The focus of this study is the mature larva of the sunflower stem weevil, *Cylindrocopturus adspersus* (LeConte), which in recent years has caused extensive damage to cultivated sunflowers in the Northern Great Plains (Charlet *et al.*, 1985). *C. adspersus* spends approx. 8 months of its life cycle as a mature larva within an overwintering chamber in the sunflower stalk or root crown and can be found overwintering in the state of North Dakota at a latitude of 47°N (Charlet, 1987). The objective of this study was to determine the metabolic and physiological compensations and adjustments that the larva of *C. adspersus* must undergo to survive the winter in North Dakota.

MATERIALS AND METHODS

Insects

Sunflower stalks from fields known to have been infested with *C. adspersus* larvae were collected in southeastern North Dakota in October, 1991. Stalks, including intact roots, were transported directly to field plots at Fargo, North Dakota where the research was performed. The stalks were replanted in the test field so that they were upright with the tap root buried in the soil. Some of the stalks were placed directly into environmentally controlled chambers maintained at either 0°C or 20°C in constant darkness. Stalks were periodically collected from the test plot and environmentally controlled chambers, split lengthwise and the larvae removed for analysis.

Temperature of crystallization

The temperature of crystallization of a larva was determined by positioning a 36-gauge copper-constantan thermocouple in contact with the insect cuticle of a surface dry larva. Temperatures were measured by a recording potentiometer (Omega multichannel recorder, Omega Engineering, Stamford, CT). A cooling rate of about 0.8°C/min was obtained by placing the larvae in a Dewar flask with a Styrofoam plug into a freezer maintained at −80°C. The temperature of crystallization was determined as the temperature recorded just prior to the rise in temperature due to the liberation of heat from the freezing of body water. Ten to 12 larvae were used to determine the mean crystallization temperature of each collection group.

Carbohydrate-polyol analysis

Potential cryoprotectant-antifreeze agents in the form of carbohydrates and polyols were screened and quantified by high-performance liquid chromatography (Waters Associates, Milford, MA) as described by Hendrix *et al.* (1981) and Lee *et al.* (1983). Samples consisted of 4 larvae per replicate and in most cases three replicates were prepared for each sampling unless otherwise specified. Preparation of the insect tissue for whole body analysis was similar to that described by Rojas *et al.* (1986). Glycogen concentration was measured using Dreywood's anthrone reagent (Morris, 1984) and tissue preparation was as described in Rojas *et al.* (1991).

Statistics

Students' t-test was used for the comparison of two means.

RESULTS

Glycogen and trehalose

Carbohydrate-polyol screening (glycerol, fructose, glucose, sorbitol, trehalose) by high performance liquid chromatography showed that the major carbohydrate present in the larvae was the disaccharide trehalose with trace amounts of glucose. The seasonal profile of whole-body trehalose and glycogen content of larvae are presented in Fig. 1 along with the average daily soil temperature at a depth of 10 cm. Larvae showed an initial steep increase in trehalose concentration from October to November [32.5 ($n = 1$) to 58.7 ± 1.2 ($n = 6$) μg/mg wet wt] with an equal reduction in glycogen [65 ($n = 1$) to 39.5 ± 1.3 ($n = 6$) μg/mg wet wt]. Then from November to January the trehalose concentration remained at a high level while the glycogen level continued to decline to 6.6 ($n = 1$) μg/mg wet wt. Larvae from the February sampling attained the highest trehalose level at 69.2 ± 2.6 ($n = 3$) μg/mg wet wt. By April, the trehalose concentration in the larvae fell to approximately that of larvae collected at the beginning of the overwintering period in October with a concomitant and equal rise in the glycogen concentration. The trend of larval trehalose accumulation from October to December coincides with falling soil temperatures during this period and the reconversion to glycogen from March to May coincides with rising soil temperatures.

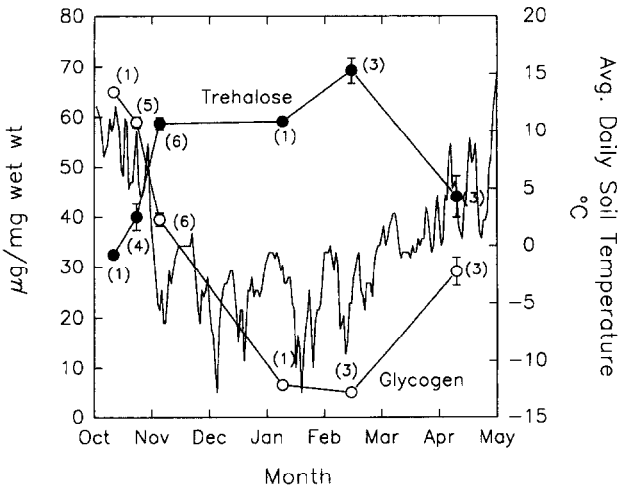

FIGURE 1. Seasonal changes in whole-body glycogen and trehalose concentrations (μg/mg wet wt) of fifth-instar larvae of *C. adspersus* from stalks kept outdoors and average daily soil temperatures at a depth of 10 cm. Glycogen and trehalose values are expressed as mean ±SEM; the number of replicates for each sampling are indicated next to its symbol.

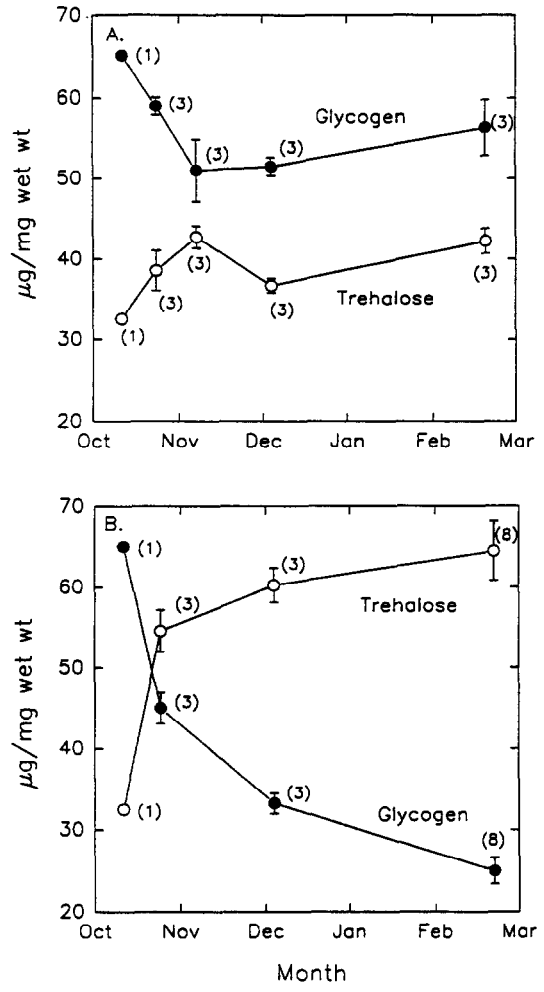

FIGURE 2. Effect of temperature acclimation on whole-body glycogen and trehalose concentrations of fifth-instar larvae of *C. adspersus* (A) 20° and (B) 0°C acclimation. Values are expressed as mean ±SEM; the number of replicates are indicated next to its symbol.

The effect of temperature acclimation on the larval concentration of trehalose and glycogen is shown in Fig. 2(A) and (B). Larvae from stems kept at 20°C exhibited a two phase pattern in trehalose accumulation; the first phase occurred from October to November and was characterized by a steep increase in trehalose [32.5 ($n = 1$) to 42.7 ± 1.4 ($n = 3$) µg/mg wet wt]. The second phase was described by a stabilizing of the trehalose concentration. The glycogen concentration fell sharply during the first phase and then leveled off during the second phase of trehalose accumulation as would be expected if trehalose is derived from glycogen reserves. However, larvae recovered from stems kept at 0°C showed a much sharper increase in trehalose from October to November going from 32.5 to 54.6 ± 2.6 ($n = 3$) µg/mg wet wt and while the rate of increase slowed by December, trehalose steadily increased to reach a maximum concentration of 64.4 ± 3.6 ($n = 8$) µg/mg wet wt by the middle of February when the experiment was terminated. Glycogen loss also closely paralleled the trehalose accumulation, falling sharply at first then decreasing more slowly.

Temperature of crystallization

As illustrated in Fig. 3, the mean temperature of crystallization for larvae collected from stems placed outdoors shows a pattern which is very similar to the seasonal pattern of the trehalose levels and the soil temperatures. From October to November the mean T_c was significantly depressed from −25.0 ± 1.3 ($n = 10$) to −28.2 ± 0.6 ($n = 8$) ($P < 0.05$) when the trehalose concentration rose sharply and soil temperatures were dropping. No significant changes occurred until March when the T_c rose from −29.1 ± 0.3 in February to −27.2 ± 0.3 ($n = 10$) ($P < 0.001$) at which time soil and air temperatures are sharply rising and trehalose is being reconverted to glycogen. The water content of the larvae remained between 65 and 67% throughout the overwintering period (data not shown).

The effect of acute temperature acclimation on mean T_c values is illustrated in Fig. 4. The only statistically significant change from the initial T_c in October (−25.0 ± 1.3) ($n = 10$) occurred in the 0°C group sampled in February where the T_c dropped to −27.8 ± 0.3 ($n = 10$) ($P < 0.05$). Also, in February pupae were found in the stalks kept at 20°C and had a T_c of −21.7 ± 2.0°C ($n = 9$).

DISCUSSION

The sunflower stem weevil larva accumulated a high level of trehalose as part of its overwintering strategy. Trehalose reached the highest level in February then fell by April to approximately that of larvae collected at the start of their overwintering period. These data suggest an interconversion between glycogen and trehalose in the larva; the glycogen concentration fell as the trehalose concentration rose with the onset of winter and conversely, the glycogen concentration rose as the trehalose concentration dropped with the onset of spring. This pattern of interconversion between glycogen and trehalose has been reported for other overwintering insects (Hayakwa and Chino, 1984; Shimada *et al.*, 1984; Rojas *et al.*, 1991). Larvae collected from the field in February had whole body trehalose levels of 69 µg/mg wet wt which to our knowledge is the highest level of trehalose reported in an overwintering insect.

Larval acclimation experiments suggest that low temperature exposure (0°C) induces glycogen catabolism and trehalose accumulation but that there is an underlying developmental factor that also results in trehalose accumulation but not to the extent as low temperature exposure. This developmental factor is evident from larvae acclimated to 20°C that showed a steep increase in trehalose levels and a concomitant drop in glycogen levels at the beginning of the overwintering period which then stabilize. However, the concentration of trehalose accumulated at 20°C is well below that accumulated by larvae collected from the field or acclimated to 0°C. Progressive accumulation of carbohydrates/polyols with increasing duration in diapause has been reported

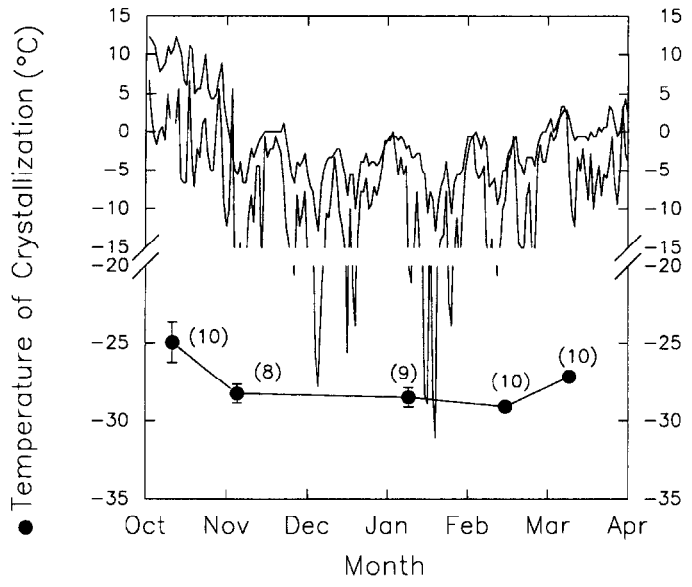

FIGURE 3. Seasonal changes in whole-body temperature of crystallization (T_cd) of fifth instar larvae of *C. adspersus* from stalks kept outdoors. Values are expressed as mean ± SEM; the sample size (n) is indicated next to each symbol. The upper tracings with no symbols are mean daily soil temperatures (10 cm below surface) (upper tracing) and low daily air temperatures (lower tracing).

in other insects and does not require additional environmental cues like low temperature. In *Sacrophaga crassipalpis* the glycerol level gradually increased during the first 40 days of diapause (from <10 mM to >70 mM) when held at a constant temperature of 20°C (Lee *et al.*, 1987). Trehalose steadily increased in diapausing pupae of *Pieris brassicae* kept at 23°C which peaked 14 days after pupation and was further enhanced by exposure to low temperature (Moreau *et al.*, 1981). The hormonal changes associated with diapause may be responsible for this accumulation of trehalose without low temperature exposure and may be secondary to the suppression of oxidative metabolism. Ecdysone injection of diapausing *P. brassicae* pupae resulted in a decline in trehalose levels and an increased respiratory rate after several days (Pullin, 1992).

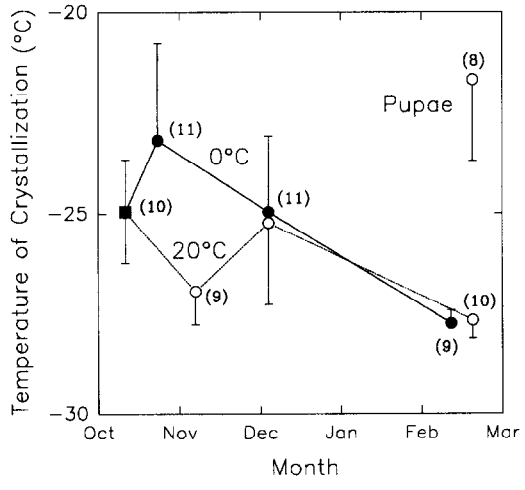

FIGURE 4. Effect of temperature acclimation (20° and 0°C) on larval whole-body temperature of crystallization (T_c). Values are expressed as mean ± SEM; the sample size (n) is indicated nest to each symbol.

Analysis of the supercooling capacity achieved by larvae collected from the field shows that larvae enter the overwintering period with a low mean T_c of approx. −25°C which drops to approx. −28°C in November. This drop coincided with the increase in whole body trehalose levels and steadily falling soil and air temperatures. The lowest mean T_c achieved (~ -29°C) was in larvae collected from the field in February when the trehalose concentration peaked. While these two parameters are closely associated with each other it is not clear whether trehalose is acting as an antifreeze in this insect since the whole body levels are not theoretically sufficient to effect these changes in the supercooling capacity. Furthermore, the larvae acclimated to both 0 and 20°C show a similar decline in the T_c by December even though whole body trehalose levels are significantly different between these two groups. Trehalose, more likely, is acting as a protectant against cold-induced changes in membranes and macromolecules and/or desiccation stress. A more thorough discussion of the possible roles of trehalose as a cryoprotectant is provided in a previous report from our laboratory [see Rojas *et al.*, 1991]; also see Wiemken (1990) for a review of trehalose as a protectant in yeast and Van Laere (1989) for a review of trehalose as a stress metabolite in various organisms.

The majority of the larvae overwinter at the base of the stalk and below the soil surface in the root crown (Rogers and Jones, 1979; Charlet *et al.*, 1985) and would experience temperatures somewhere between the extreme lows of the air and those 10 cm below the soil surface. It can be seen from Fig. 3 that the larvae would not be

at risk of spontaneous freezing throughout the overwintering period and avoidance of freezing through their extensive supercooling capacity may be integral to their overwintering strategy. Further study is required to determine the lower lethal temperature limits for this species and its relationship to larval supercooling capacity.

C. adspersus shares some similar attributes of its overwintering physiology and biochemistry to the red sunflower seed weevil, *Smicronyx fulvus*, which also overwinters in North Dakota but exposed in the top 10 cm of the soil surface. Both these insects accumulate trehalose from glycogen stores as part of their overwintering strategy with peak levels occurring in the middle of winter (Jan–Feb). However, the sunflower stem weevil larva achieves a maximum level of 69 μg/mg wet wt while the seed weevil larva achieves maximum of 40 μg/mg wet wt. Similarly, the larva of *C. adspersus* has a higher level of glycogen than the sunflower seed weevil larva at the beginning of the overwintering period (65 vs 18 μg/mg wet wt). The T_c of both these insects is low at the start of the overwintering period (~ -25°C). However, while the T_c remains constant in the sunflower seed weevil, it drops to a low of -29°C in February in the sunflower stem weevil (Rojas *et al.*, 1991). Also, *C. adspersus* larvae maintained a much higher water content ($\sim$66%) than the sunflower seed weevil larvae ($\sim$40–45%) while overwintering.

Preliminary studies from our laboratory indicate that several other sunflower pests overwintering in North Dakota also accumulate trehalose and extensively supercool. This raises the questions of whether the production of high levels of trehalose is metabolically linked to the host plant and what mechanism allows for such high levels of what is normally a tightly regulated blood sugar to be accumulated by these insects.

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